

# Evaluation of the immunomodulatory effects of undecafluoro-2-methyl-3-oxahexanoic acid (“GenX”) in C57BL/6 mice

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**Abstract:** Undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA), known as “GenX” by its U.S. manufacturer, is a compound designed to replace perfluorooctanoic acid (PFOA), a perfluoroalkyl compound that has been phased out of U.S. production due to environmental persistence, detectable serum concentrations in humans and wildlife, and reports of systemic toxicity. In experimental rodent models PFOA exposure suppresses T cell-dependent antibody responses (TDAR) and has been reported to suppress vaccine responses in exposed humans. To determine if U2M3-OHxA also modulates TDAR, male and female C57BL/6 mice were exposed

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to 0, 1, 10, or 100 mg/kg of U2M3-OHxA by gavage for 28 days. Serum and urine were collected 24, 48, 72 hours, 5 days, and 14 days after the initial dose to determine U2M3-OHxA concentrations. Mice were immunized with sheep red blood cells (SRBCs) on day 24, SRBC-specific IgM antibody responses and splenic lymphocyte subpopulations were evaluated five days later. Body weight did not differ by dose but relative liver weight increased in both sexes at the 10 and 100 mg/kg doses. SRBC-specific IgM antibody production was suppressed in male and female animals exposed to 100 mg/kg. No changes in splenic T lymphocytes were observed for either sex, but B lymphocytes were suppressed at 1 and 100 mg/kg in male animals, which has not been observed in animals exposed to PFOA or PFOS. While this PFOA-replacement compound appears to be less potent at suppressing TDAR relative to PFOA, further studies are necessary to determine its full immunomodulatory profile and its potential synergism with other per- and polyfluoroalkyl compounds of environmental concern.

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## Introduction

Per- and polyfluoroalkyl acids (PFASs) are anthropogenic organic compounds comprised of strong carbon-fluorine bonds that make them extremely useful as polymerization aids and surfactants for the processing of myriad consumer and industrial products. However, the characteristics that make these compounds beneficial in industrial processes make them problematic from an environmental health standpoint; they persist in the environment and some bioaccumulate ~~bio accumulate~~ in wildlife and humans. They also have been associated with multisystem toxicity, most notably immunotoxicity, as similar effects on the immune system have been observed in both exposed experimental animal models and humans. Among the most well characterized PFASs are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), which have been reported to potently suppress T cell-dependent antibody responses (TDAR) in experimental rodent models and responses to vaccinations in exposed humans. Largely due to environmental persistence and growing reports of toxicity, PFOS was phased out of production by its major manufacturer in 2002 and PFOA was phased out of production by its major U.S. manufacturers in 2015. However, their usefulness as processing aids has prompted research into alternative PFASs with chemical characteristics that will reduce their persistence and toxicity.

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The search for PFOA alternatives has focused on shorter-chain PFASs based on the assumption that they are less persistent and toxic than their longer chain counterparts (Stahl et al., 2011). One replacement is a six-carbon compound known as undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) or 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoic acid ( $C_6H_9F_{11}O_3$   ~~$C_6H_{11}O_3$~~ ), with the trade name “GenX” (**Figure 1**). It was introduced to fluoropolymer manufacturing processes in 2009 by a major U.S. manufacturer. Data from publicly available

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documents associated with a National Pollutant Discharge Elimination System (NPDES) permit at one of its manufacturing facilities indicates a “favorable toxicological profile.” Industry studies referred to in the NPDES permit documentation list a no observed adverse effect level (NOAEL) of 10 mg/kg based on evidence of regenerative anemia in male rats exposed to 100 mg/kg and female rats exposed to 1000 mg/kg for 90 days; the exposure route and strain of rat was not specified in the document. At the NOAEL concentration, male rats also had a reduced red blood cell count, hematocrit, and hemoglobin. To our knowledge, no peer-reviewed published information on its toxicological profile exists. Our goal in undertaking this initial study was to determine serum and urine concentrations of U2M3-OHxA as well as its potential to affect the TDAR with or without concomitant effects on splenic lymphocyte subpopulations in mice as a way to compare its immunomodulatory potential to that of PFOA. The current study generally followed the U.S. Environmental Protection Agency’s (EPA) Immunotoxicity Harmonized Test Guideline (OPPTS 870.7800) to assess the potential immunotoxicity of U2M3-OHxA.

## **Materials and Methods**

**Animals:** A total of 96 C57BL/6 mice in two replicates of 48 animals each, spaced about eight weeks apart, were used for this experiment and were obtained from Charles River Laboratories (Raleigh, NC) at 7 weeks of age. Upon arrival to AAALAC accredited animal facilities at East Carolina University (ECU), mice were housed in groups of three and separated by sex. Distribution of animals from shipping containers was done semi-randomly, with the first animal being added to the first cage, the second to the second cage, the third to the third cage, and so forth. After distribution into cages, animals were weighed and redistributed to different

cages to equalize body weights among cages so that pre-dosing body weights did not differ statistically by cage within sex ( $p < 0.05$ ). Food and water were provided *ad libitum*, and animals were kept on a 12-hour light-dark cycle, 20-24°C, and a relative humidity of 60-65%. Bedding was changed twice weekly and the health of mice was monitored daily by both researchers and animal care staff. Mice were given five days to acclimate to their new housing arrangements before dosing began and the ECU Institutional Animal Care and Use Committee approved all procedures in advance.

**Dosing:** Mice were randomly assigned by cage to four different dose groups with six males and six females per group and two cages/sex/group housing three animals each. Doses were selected based on the reported NOAEL from the rat data extracted from the NPDES permit and reduced by an order of magnitude due to uncertainty about the sensitivity of mice relative to rats. Doses therefore were 0, 1, 10, or 100 mg/kg. The test chemical (CAS# 13252-13-6) was acquired from Synquest Laboratories (Alachua FL, USA). U2M3-OHxA solution was prepared fresh at the beginning of each week with sterile water and 0.5% Tween-20 vehicle to ensure emulsification into the dosing water. Concentrations of U2M3-OHxA dosing solutions were 0, 0.1, 1, and 10 mg/mL, which when given at 0.1 µL/g of body weight resulted in the appropriate mg/kg dose. Each mouse was dosed daily via oral gavage for 28 days. Body weights and deviations from normal appearance suggestive of systemic toxicity (i.e., piloerection, hunched posture, failure to move appropriately) were recorded daily before dosing.

**Organ Endpoints:** One day after the final gavage exposure, mice were humanely euthanized via IACUC-approved methods and necropsied. Spleen, liver, and thymus were harvested and weighed immediately. Livers were stored at -80°C for later analysis of peroxisome proliferation, spleens were stored on ice in 3 mL of complete medium (RPMI, 10% fetal calf

serum, 50 IU penicillin and 50 µg streptomycin), and thymuses were discarded after weights were recorded. Livers were homogenized and acyl-CoA oxidase, as a measure of hepatic peroxisomal fatty acid oxidation, was assayed fluorimetrically with pamitoyl-CoA as a substrate as per Poosch and Yamazaki (1986).

***Immunophenotyping:*** Spleens were aseptically processed into single-cell suspensions by gentle grinding, passage through a 70 µm nylon filter, and followed by the addition of 7 mL of complete medium. An aliquot of each spleen suspension was counted on a Nexcelom Bioscience Cellometer Auto 2000 cell counter (Nexcelom Bioscience LLC, Lawrence, MA) to determine the number of live cells. The total number of cells per spleen (cellularity), adjusted by the weight of each organ, was determined for each animal. Suspensions were adjusted to  $2 \times 10^7$  cells/mL. Optimal concentrations of flow antibodies, reagents, and isotype controls to estimate non-specific binding were determined in previous experiments (DeWitt et al., 2015). All experimental replicates also included unstained cells as negative controls and single color controls as positive controls to determine color compensation. Monoclonal antibodies (eBioscience, Inc., San Diego, CA) coupled to fluorochromes specific for the following markers were used: anti-mouse APC anti-mouse CD3e, FITC anti-mouse CD4, PE anti-mouse CD8a, and FITC anti-mouse CD45RB. Flow cytometric analysis was performed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and 10,000 events were collected from each sample. The total number of each cell type was determined from spleen cellularity.

***IgM Antibody Response:*** On the 24<sup>th</sup> day after the initial dose, mice were immunized with sheep red blood cells (SRBCs) via tail vein injections. SRBCs were adjusted to  $4 \times 10^7$  cells in 0.2 mL of sterile saline. Sera for measurement of SRBC-specific IgM antibodies was collected as described previously. IgM antibody titers were determined as described by DeWitt et al. (2015).

Briefly, flat bottom 96-well Immunolon-2 ELISA microtiter plates (Dynatech Labs, Chantilly, VA) were coated with 125  $\mu$ l of 2  $\mu$ g/ml of SRBC membrane [1.46 mg/ml stock solution diluted in phosphate-buffered saline (PBS); prepared according to Temple et al. (1995)] and then incubated at 4°C for at least 16 hr. Each plate included 20 wells coated with pooled serum collected from healthy mice 5 d after primary immunization with SRBC, and 16 wells contained 100  $\mu$ l PBS as blanks. After washing, blocking of non-specific binding, and addition of serum samples (serially diluted 1:2, starting at 1:8), secondary antibody (goat anti-mouse IgM horseradish peroxidase; Accurate Chemical and Scientific Corp., Westbury, NY) was added to the wells. Following three washes and addition of substrate [10 mg 2,2'-azino-di-(3 ethylbenz-thiazoline sulfonic acid, ABTS, Sigma) added to 50 ml phosphate-citrate buffer with one tablet of urea hydroxide peroxide (Sigma) in 100 ml distilled water, 0.05 M final solution], plates were incubated for 45 min at room temperature and then the absorbance in each well evaluated at 410 nm on a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). IgM anti-body titers were processed using SOFTmax Pro software (Molecular Devices, LLC, Sunnyvale, CA) to determine the log<sub>2</sub> serum titers for an optical density of 0.5 U from the log-log curve of optical density versus dilution, as described by Temple et al. (1995).

***Sera and Urine U2M3-OHxA Concentrations:*** Blood was collected 24 hours, 5 days, 14 days, and 29 days after the initial dose. Prior to the terminal bleed at day 29, blood was collected via the submandibular vascular bundle; blood at terminus was collected via neck vein transection from anesthetized animals. Blood was allowed to clot at room temperature for 30 minutes, serum was separated from the clot by centrifugation at 4°C, and then frozen at -80°C for later analyses. Urine was collected non-invasively by placing each of three animals/cage into a clean cage free of bedding and collecting deposited urine. This pooled urine was collected 24, 48, 72 hours, 14

days, and 29 days after exposure began. Sera and urine samples from each time point were evaluated with some fancy-assed equipment run by the brilliant scientist Mark Strynar. His shit is good. Believe it, using the methods of [Reiner et al., 2009] developed for PFOA analysis with minor modifications. In brief serum and urine samples were denatured with a solution of 0.1M formic acid followed by an acetonitrile protein crash. Constructed standard curves were created using blank CD1 mouse serum (Pel Freez Biologicals Rogers, AR USA) or control animal urine depending on the matrix. Analyte in dosed and control serum/urine were quantitated using the stable isotope dilution method with  $^{13}\text{C}_2$ -perfluorohexanoic acid purchased from Wellington Labs (Guelph, Ontario Canada) as an internal standard. At the time of this analysis not stable isotope labeled GenX was available. Calibration curves, process blanks, matrix blanks and replicate samples were run with every assay to maintain quality control.

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Jessica L. Reiner, Shoji F. Nakayama, Amy D. Delinsky, Jason P. Stanko, Suzanne E. Fenton, Andrew B. Lindstrom, Mark J. Strynar  
Reproductive Toxicology 27 (2009) 360–364

**Statistical Analysis:** Data are presented as mean  $\pm$  standard deviation unless otherwise indicated and all statistical analyses were performed with the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data from the replicates were evaluated for homogeneity and combined as no heterogeneity was detected within sex and within dose between replicates. Therefore presented endpoints reflect N = 12 animals/sex/dose. Serum and urine concentrations were determined from only one experimental replicate (N = 6 animals/sex/dose). However, due to low blood collection volumes from individual animals at individual time points, serum concentrations reflect N = 1-6; concentrations based only on N = 1 are reflected in figure legends. Urine samples reflect pooled samples of three animals/cage and two pooled samples/dose as animals were housed three/cage with two cages/dose. Urine samples, therefore reflect N = 1 or 2 depending on how much urine was non-invasively excreted by animals on a particular collection day. Body weights over the course of the study were evaluated by two-way



analysis of variance (ANOVA) with a repeated factor; all other data were evaluated separately within sex and across dose by one-way ANOVA with appropriate post-hoc t-tests when the overall ANOVA revealed an F-statistic with a p-value < 0.05.

## Results

### *Body and Organ Endpoints*

Body weight did not differ by dose within sex over the course of exposure or at experimental terminus (**Figure 2**), nor did animals show outward signs of systemic toxicity. Absolute (data not shown) and relative (**Figure 3**) liver weights were increased ( $p < 0.0001$ ) by 40-160% in both males and females exposed to 10 or 100 mg/kg, with males having a greater percent increase relative to the 0 mg/kg group compared to females. Absolute and relative thymus weights did not differ statistically by dose within either sex; absolute and relative spleen weights from females exposed to 100 mg/kg were 16.5% and 11.3% lower ( $p < 0.002$ ) compared to weights from the 0 mg/kg group (**Table 1**). Splenic lymphocyte cellularity was increased by 63.5% ( $p < 0.04$ ) in males exposed to 100 mg/kg relative to the 0 mg/kg group; no other differences in splenic lymphocyte cellularity were noted. This difference was likely due to one male in the 100 mg/kg group having a larger spleen (115 mg compared to an average of 91.5 for the remaining spleens) with a larger number of lymphocytes ( $95.3 \times 10^6$  compared to an average of  $57.9 \times 10^6$  for the remaining spleens). This animal was the largest animal in the group, but only by 0.1 g and did not show signs of having an infection or other health issue. Peroxisomal fatty acid oxidation also was increased in both males and females (**Figure 4**). Hepatic acyl-CoA oxidase activity was increased 122% and 222% in livers of male animals exposed to 10 or 100

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mg/kg of U2M3-OHxA, respectively ( $p < 0.03$ ). In female animals, only exposure to 100 mg/kg statistically increased acyl-CoA oxidase activity (by 100%;  $p < 0.02$ ).

### ***Immunophenotyping***

In both males and females, splenic T-cell subpopulations were not altered in any of the dose groups (**Table 1**). Splenic B-cell percentages, however, were statistically reduced by U2M3-OHxA exposure. The percentage of B-cells in males exposed to 1 or 100 mg/kg was reduced ( $p < 0.01$ ) by 5.7% and 10.1% respectively (**Table 1**). The percentage of B-cells in females were not statistically altered by exposure.

### ***IgM Antibody Responses***

SRBC-specific IgM antibody responses were suppressed ( $p < 0.009$ ) by 7.3% in females exposed to 100 mg/kg, whereas males did not experience any statistically significant differences in IgM antibody production (**Figure 5**).

### ***Serum and Urine Concentrations***

Relative to the 0 mg/kg group, serum concentrations of U2M3-OHxA were statistically elevated at all time points evaluated (after 1, 5, 14, and 28 days of exposure) in animals exposed to 10 or 100 mg/kg ( $p < 0.05$ ; **Figure 6**). In both males and females exposed to 10 mg/kg, serum concentrations dropped by about 50% from five to 14 days of exposure and in females, serum concentrations dropped again by about 40% from 14 days of exposure to one day after exposure ended (terminus). In males exposed to 10 mg/kg, serum concentrations dropped only by about 10% from 14 days of exposure to one day after exposure ended. In animals exposed to 100 mg/kg, maximal concentrations were reached after five days of exposure. In males, this was maintained until after 14 days of exposure whereas in females, serum concentrations, similarly to the 10 mg/kg group, dropped by about 50% from five to 14 days of exposure and by about 20%

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from 14 days of exposure to one day after exposure ended. In males, serum concentrations dropped by about 30% from 14 days of exposure to one day after exposure ended.

As full urine samples were not available on each day for all groups due to lack of urine excretion, it is challenging to meaningfully interpret urinary excretion trends. What is abundantly clear from the urine data, however, is that males excreted a much higher concentration of U2M3-OHxA across all concentrations and time points collected relative to females (**Figure 7**).

## Discussion

U2M3-OHxA is a compound that was designed to replace PFOA in various manufacturing processes and is marketed as a processing aid with a toxicological profile that is more favorable than PFOA and that has rapid bioelimination. It was introduced to fluoropolymer manufacturing processes in 2009 and to our knowledge no peer-reviewed published data about its toxicological effects exist. However, a recently published study highlighted its absorption, distribution, metabolism, excretion, and kinetics in rats, mice, and cynomolgus monkeys following a single dose (Gannon et al., 2016). In one experiment presented in this study, Sprague-Dawley rats of each sex and Crl:CD1 (ICR) mice of each sex were given a single oral dose of 10 or 30 mg/kg of the ammonium form of U2M3-OHxA ( $\text{C}_6\text{H}_{14}\text{O}_3\text{H}_3\text{N}^+\text{C}_6\text{F}_{11}\text{O}_3^-\text{NH}_4^+$ ) and blood samples were collected at various times within the first 24 hours following administration of the test substance and then once daily for seven days; urine samples also were collected during this time. Biphasic kinetics were observed in both species, with mice having slower plasma elimination than rats and female animals having more rapid plasma elimination, overall, compared to male animals (Gannon et al., 2016). Urine data indicated rapid and complete elimination without metabolization (metabolism) (Gannon et al., 2016). In another experiment presented in this study,

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male and female Sprague-Dawley rats and cynomolgus monkeys were given a single intravenous dose of 10 mg/kg (rats and monkeys) or 50 mg/kg (rats only). Biphasic kinetics also were observed in both species, with plasma elimination being similar to the orally administered dose (Gannon et al., 2016). The authors concluded that the test substance is more rapidly eliminated compared to PFOA and that given the similarities between rats and monkeys, rats may be an appropriate model of pharmacokinetics in primates (Gannon et al., 2016).

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In our study, we chose the C57Bl/6 mouse model as it is a rodent model that is highly sensitive to the immunomodulatory effects of PFOA exposure. We also chose to evaluate the U2M3-OHxA compound following a standard immunotoxicity testing protocol, employing a 28-day exposure duration and evaluating antigen-specific antibody responses (i.e., TDAR) and enumeration of splenic lymphocytes. TDAR also was chosen as suppression of the TDAR has been reported in various rodent species after exposure to ammonium perfluorooctanoate (APFO, the ammonium salt precursor of PFOA), PFOA, or PFOS (DeWitt et al., 2008, 2009, 2016; Loveless et al., 2008; Peden-Adams et al., 2008; Yang et al., 2002) and in humans as reduced responses to vaccine administration (Grandjean et al., 2012; Granum et al., 2013; Looker et al., 2014). In addition, we evaluated peroxisome proliferation, which is a biomarker of PFOA exposure, general markers of toxicity, and measured serum and urine concentrations over the course of the study. Therefore, our major goal was to compare the results of this study to similar studies of PFOA to attempt to discern if U2M3-OHxA produces immunotoxicological effects similarly to or differently from PFOA.

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No statistically significant effects on body weights were observed nor were any overt signs of systemic toxicity noted in our study at doses of U2M3-OHxA up to 100 mg/kg given by gavage for 28 days. In contrast, when female C57Bl/6 mice were given 30 mg/kg of PFOA in

drinking water for 15 days, body weight was statistically reduced starting at eight days of exposure through the end of the study when compared to body weights of control animals (DeWitt et al., 2008). In the same study, mice given 15 mg/kg of PFOA had reduced body weight relative to the control animals by the end of the study (DeWitt et al., 2008). Loveless et al. (2008) reported body weight loss in male Crl:CD-1(ICR)BR given 10 or 30 mg/kg APFO via gavage for 29 days as did Yang et al. (2001) in male C57Bl/6 mice given 40 mg/kg of PFOA in the diet for 10 days. When strictly compared on a dose basis, it appears as if a much higher dose of U2M3-OHxA (100 mg/kg versus 10 – 40 mg/kg) was not sufficient to reduce body weights of male or female C57Bl/6 mice, even when given for a longer exposure period (28 days versus up to 29 days).

Exposure to U2M3-OHxA increased both absolute and relative liver weights. Both males and females exposed to 10 or 100 mg/kg exhibited liver weight increases compared to liver weights of animals exposed to 0 mg/kg. However, exposure to 1 mg/kg did not result in increased liver weights. Similar doses of PFOA do result in increased liver weights; exposure to either 0.94 or 1.88 mg/kg of PFOA via drinking water for 15 days increased absolute and relative liver weights in female C57Bl/6 mice when evaluated at the end of the study (DeWitt et al., 2008). A higher dose of PFOA, 40 mg/kg, was reported to increase liver weights in male C57Bl/6 mice after only two days of exposure (Yang et al., 2000) and Loveless et al. (2008) reported liver weight increases in male Crl:CD-1(ICR)BR given APFO for 29 days. Acyl-CoA oxidase activity, a marker of hepatic peroxisome proliferation, was increased in both males and females exposed to 100 mg/kg and in males exposed to 10 mg/kg. An earlier study on the potential immunotoxicity of PFOA reported increases in hepatic acyl-Co-A at a low dose of about 2 mg/kg after 10 days of dietary exposure in male C57Bl/6 mice (Yang et al., 2001). In

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terms of liver endpoints typically evaluated after PFOA exposure, it appears as if U2M3-OHxA is not as potent in inducing either hepatomegaly or hepatic peroxisome proliferation as PFOA.

Lymphoid organs also appeared to be less sensitive to U2M3-OHxA exposure than to PFOA exposure. The high dose (100 mg/kg) of U2M3-OHxA was associated with a reduction in absolute and relative spleen weights in female animals whereas PFOA and APFO have been reported to reduce both spleen and thymus weights at lower doses (10 – 40 mg/kg) and after shorter or similar exposure durations (DeWitt et al., 2008; Loveless et al., 2008; Yang et al., 2000, 2001). It is probable, given the weight loss associated with these relatively higher doses of PFOA, that lymphoid organ atrophy occurred as a result of systemic toxicity rather than immune system-specific effects of PFOA exposure.

The effect of PFOA exposure on antigen-specific antibody production has been documented in several strains of mice and is supported by epidemiological studies that associated serum PFOA concentrations with reduced responses to vaccines. These data are particularly robust as there is agreement among the studies, although interpretation of the results varies across studies. The TDAR is a measure of immune function that is highly predictive of the ability of an agent to induce immunotoxicity in exposed humans if it is altered in experimental animal models (Luster et al., 1992). The lowest observed adverse effect level (LOAEL) reported for female C57Bl/6 mice given PFOA via drinking water for 15 days was 3.75 mg/kg, with a benchmark dose of 3.06 mg/kg (DeWitt et al., 2008). Loveless et al. (2008) revealed a LOAEL of 10 mg/kg in male Crl:CD-1(ICR)BR mice given APFO via gavage for 29 days and Yang et al. (2002a) reported suppression of the TDAR in male C57Bl/6 mice fed 40 mg/kg of PFOA for 10 days. Loveless et al. (2008) suggested that this suppression of the TDAR was a result of systemic toxicity rather than immune system-specific effects of PFOA or APFO exposure. Doses of APFO

or PFOA  $\geq 10$  mg/kg have been associated with statistically significant reductions in body weight, a sign of systemic toxicity. Loveless et al. (2008) reported a negative correlation between serum corticosterone, a measure of systemic toxicity and stress, and the TDAR in APFO-exposed mice and as no reduction in body weight or the TDAR was correlated with increases in serum corticosterone at APFO doses of 0.3 or 1 mg/kg, they concluded that APFO was not immunotoxic at their tested doses. In a follow-up study, DeWitt et al. (2009) exposed adrenalectomized (adx) C57Bl/6 female mice to PFOA via drinking water for 10 days and reported suppression of the TDAR at a dose of 7.5 mg/kg, which was not associated with body weight loss or elevated corticosterone production in the adx animals. While systemic toxicity and stress cannot be discounted as factor contributing to the immune effects of PFOA in mice given relatively high doses ( $\geq 10$  mg/kg), it does not appear to be a factor in the immune effects of PFOA given at lower doses. In terms of the effects of U2M3-OHxA on the TDAR, suppression of this response was observed only in female animals exposed to 100 mg/kg. This suppression was relatively mild; IgM production was 7.3% lower in treated animals relative to control animals. This suppression also was not associated with a reduction in body weight or overt signs of systemic toxicity. Relative to PFOA, U2M3-OHxA appears to be much less potent at affecting the TDAR.

Of particular interest regarding the effects of PFOA on the TDAR is that IgM antibody suppression occurs at doses that do not produce notable effects on splenic lymphocyte numbers as well as T and B cell subtypes. Higher doses of PFOA and APFO have been associated with reduced splenic and thymic lymphocyte numbers (Loveless et al., 2008; Yang et al., 2000, 2001) as well as reductions in T and B cell subtypes (Yang et al., 2001). However, lower doses of PFOA have not been reported to consistently alter lymphocyte numbers in lymphoid organs or T

and B cell subtypes (DeWitt et al., 2016). In this study, U2M3-OHxA exposure failed to alter splenic lymphocyte numbers or T cell subpopulations when compared to control values. In male animals, however, the percentage of B cells was reduced by exposure to 1 or 100 mg/kg of U2M3-OHxA. The reductions were mild, 5.7% and 10.1%, and were not observed in female animals, even at the dose that was associated with a reduction in the TDAR. These data suggest that U2M3-OHxA may affect the TDAR via different pathways than does PFOA. Several studies have attempted to determine the mechanism(s) by which PFOA impacts the TDAR and to date, no consensus has been demonstrated by the data. Some studies (Yang et al., 2002b; DeWitt et al., 2016) have attempted to link peroxisome proliferation via activation of the peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ) with PFOA-induced suppression of T and B cell subpopulations or the TDAR, but even though PPAR $\alpha$ -null animals have somewhat attenuated responses to PFOA, they still exhibit suppression of responses. Other studies have included in vitro and ex vivo assays to evaluate cytokine production following PFOA exposure, but no studies have specifically associated changes in cytokine production with suppression of the TDAR. Additional studies with both PFOA, U2M3-OHxA, and additional PFASs are warranted to determine the mechanism(s) associated with suppression of the TDAR and whether or not the mechanism is associated with chain length, functional group, per- or poly-substitution, or other factor.

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To our knowledge, Gannon et al. (2016) is the only published study of pharmacokinetics of U2M3-OHxA. As previously discussed, Gannon et al. (2016) reported U2M3-OHxA is cleared from plasma more quickly in female mice than in male mice and that urinary excretion is rapid and complete. Our data support this general trend although our study was not conducted according to pharmacokinetic study guidelines. In our study, male animals had much higher



serum and urine concentrations as the study progressed relative to female animals, suggesting slower plasma and therefore urinary clearance. Relative to PFOA, and in support of the Gannon et al. (2016) study, it appears as if U2M3-OHxA has a shorter serum half-life and more rapid excretion, at least in mice. Unlike PFOA, it appears as if U2M3-OHxA is accumulated and excreted at a different rate in male and female mice; pharmacokinetic studies and serum measures of PFOA suggest that male and female mice accumulate and excrete PFOA at similar rates. Future studies with mice exposed to U2M3-OHxA should therefore evaluate toxicological endpoints in both sexes.

**Commented [SM15]:** I am not sure if this is true. I think Lau showed PFOA elimination in the CD1 mouse was different in the male and female, however it was no way near the differences observed in the dosed Sprague-Dawley rats. I know the CD1 mouse had a gender difference with PFOA. I just don't recall if it was statistically significant.

## Conclusions

Oral U2M3-OHxA exposure in C57Bl/6 mice, unlike PFOA, is not a potent suppressor of the TDAR, even at doses that would induce high mortality for PFOA. Effects on immune parameters, liver weights, and peroxisome proliferation, while less robust than PFOA, were still detectable, indicating that U2M3-OHxA is a less toxic alternative to PFOA on these particular parameters. At first glance, this may seem sufficient evidence to conclude that U2M3-OHxA is a suitable replacement for PFOA. However, this study evaluated only a few endpoints, and the data suggest that U2M3-OHxA may differ from PFOA in its mechanism of action on the immune system. Additionally, given the absence of data about levels of U2M3-OHxA in environmental media, wildlife, and humans, the doses used in this study may not reflect actual exposures and there may be over- or underestimating exposure concentrations. While the shorter chain length and polyfluorinated makeup of U2M3-OHxA may be associated with fewer adverse effects than PFOA at similar doses, additional data are necessary before it can be declared a safer or less toxic alternative to PFOA.

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## References

**Commented [SM16]:** Add the Gannon reference.

Add the Reiner et al. ref from the methods section.

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